

Absolute measurement centrifuge

Related applications and patents

This is a continuation-in-part of Application number 10/202,777 filed 24 July 2002.

The following patents and applications relate to the methods of light scattering for the measurement of molecular and particle mass and size.

P. J. Wyatt, U. S. Pat. No. 6,411,383 B1 (25 June, 2002) "Method for measuring the 2nd virial coefficient."

S. Trainoff and P. J. Wyatt, Application No. 10/205,637 filed 24 July 2002 "Method for determining average solution properties of macromolecules by the injection method."

Background

Of all the devices that may be used for measuring the sizes of particles in the nanometer range, the disk and ultra centrifuges are among those most capable of providing high-resolution separations. Despite such resolution capability, the operation of such centrifuges is generally fraught with considerable ambiguities. Most of these problems are associated with uncertainties in the derived sizes of particles since such sizes are based entirely upon the arrival times of the separated particles at the detector. By using a mixture of the unknown sample particles with particles whose sizes are precisely known, these arrival times may be calibrated to some extent. Unfortunately, despite such calibrations, small variations in temperature and rotor speed, in addition to so-called streaming phenomena, often render such calibrations questionable. Another major difficulty relates to the need to know precisely the

1 density of the assumed homogeneous spherical particle structures and that of the fluid
2 environment in which the separation is performed. Whenever a gradient is used, the explicit
3 density variation should be known as well. Other problems associated with determining
4 particle size by measuring times-of-arrival at the detector include deviations of Reynolds'
5 number in excess of 0.5%, effects of sample dispersion due to Brownian motion resulting in
6 the spreading out of the arrival times of identical particles, band broadening dependent on the
7 speed of separation, establishing suitable gradients to prevent streaming, overloading sample
8 concentration, range of particle sizes in the sample, problems with deconvolution analyses,
9 *etc.* Virtually all these difficulties are associated with one basic shortcoming of these devices:
10 centrifugal separation is not an absolute measurement method for most classes of particles. In
11 other words, with the exception of a theoretical arrival time for homogeneous spheres at the
12 detector, once a set of particles has arrived, their size cannot be measured directly. Of course,
13 if the particles are not homogeneous spheres, *i. e.* of unknown structure, even the best of
14 prior calibration procedures can result in great uncertainties in interpretation.

15
16 Centrifugal separation would appear ideally suited for the subsequent application of a
17 multiangle light scattering, or MALS, analysis were it not for the inaccessibility of the
18 samples. Thus, using cross flow field flow fractionation as described by Wyatt, for example,
19 in his 1998 article "Submicrometer particle sizing by multiangle light scattering following
20 fractionation," that appeared in *J. Colloid and Interface Science* volume **197**, pages 9—20,
21 multiangle light scattering analyses of the eluant samples following separation produces
22 detailed and accurate size and distribution information. The concept has been applied also to
23 the analyses of samples separated by other methods including size exclusion chromatography

1 and capillary hydrodynamic fractionation, to name a few. A centrifugal device with an
2 accessible eluting sample following separation was developed by J. Calvin Giddings and is
3 referred to as sedimentation field flow fractionation, or SdFFF for short. This method,
4 described, for example by Giddings in his 1993 paper in volume **260** of *Science* at pages
5 1456 *et seq.*, required an elaborate set of slip rings and capillaries. Other types of FFF
6 separation techniques are also discussed in Giddings' paper. Combined with MALS, the
7 analysis of eluting samples permitted the accurate characterization of each eluting fraction of
8 particles independent of diffusion effects. Nevertheless, the SdFFF device had neither the
9 resolution nor dynamic range of the more conventional centrifugal separation devices and
10 was prone to leaks within a short time of installing new seals.

11

12 Results derived from the more conventional disk centrifuge and analytical centrifuge devices
13 are based on the optical examination of small regions within the sample volume being
14 subjected to centrifugal forces. Remote light sources, *i. e.* stationary relative to the spinning
15 samples, are synchronized to the motion of the sample through the incident light beam to
16 yield some measure of particle presence in the particular region being "interrogated." These
17 may include absorption and forward scattering measurements as well as fluorescence for
18 some devices. From such measurements, further attempts are usually made to derive a size
19 distribution of the particle sample by interpreting the scattering and/or obscuration of the
20 transmitted light beam at the detector in terms of Lorenz-Mie scattering theory, *i. e.* assuming
21 the particles are homogeneous spheres. The forward-scattered light intensity is assumed to
22 arise because such spheres of a known radius, a , have entered the incident light beam.

1 However, such “known” size was extracted from the time of arrival of the particles based on
2 the relation

$$D \approx \frac{\sqrt{18\eta \ln(R/R_0)}}{\omega(\rho_p - \rho_f)^{\frac{1}{2}} t^{\frac{1}{2}}}, \quad (1)$$

5 where $D = 2a$ is the particle diameter, ω is the angular velocity of the rotor, R_0 is the radius at
6 which the sample particles were injected at time $t = 0$, R is the radius at which they are
7 detected, η is the fluid viscosity, and ρ_p and ρ_f are the particle and fluid specific gravities,
8 respectively. Possible sources of error in the terms of Eq. (1) can be significant. Most
9 importantly, Eq. (1) only applies strictly for the case of homogeneous spherical structures.

11 Perhaps the greatest source of error in deriving particle size from Eq. (1) occurs when the
12 particle density is close to that of the medium which is the case, for example, for proteins and
13 a variety of particles produced by emulsion polymerization. When ρ_p and ρ_f are very close,
14 slight errors in ρ_p can result in significant errors in the derived particle diameter, D . In
15 addition, of course, Eq. (1) applies only to spherical particles. For non-spherical particles, the
16 hydrodynamic radius, r_h , derived is just that of an equivalent sphere. It is an objective of this
17 invention to provide a means by which the hydrodynamic radius of a particle passing through
18 the detection beam may be determined far more accurately and without reference to a known
19 particle standard, often used for centrifuge calibration. In addition to a measurement of the
20 hydrodynamic radius, a particularly useful objective of this invention is the measurement of
21 the so-called mean square radius. Knowledge of both of these radii often permits the
22 derivation of the particle structure as well.

1

2 It is a further objective of this invention to provide an absolute measure of the radius of a
3 spherical particle in the range of about 10 through 1000nm without calibration. An additional
4 objective of this invention is to permit the accurate derivation of the particle size distributions
5 of particles separated by centrifugal means even in the presence of significant diffusion
6 caused by Brownian motion. Another objective of this invention is to circumvent, whenever
7 possible, distortions in derived size distributions caused by other effects that tend to broaden
8 the separated particle bands that appear at the detector such as systematic variations in rotor
9 speed, changes in fluid temperature and viscosity, *etc.* Still another objective of this invention
10 is the ability to measure sizes and size distributions for a broad range of inhomogeneous
11 particles whose individual density variations may not be known *a priori*. Because some
12 implementations of the disk and ultracentrifuges purport to be able to measure the
13 concentration of very small particles directly, another objective of this invention is to be able
14 to measure the molar mass of certain classes of molecules separated by centrifugal means.
15 The success of the present invention to achieve these objectives depends critically upon the
16 ability to integrate a MALS detection system into a centrifugal separation device and to use
17 the existing features of centrifugal devices to permit more accurate analyses of the measured
18 samples.

19

20 **Brief descriptions of the drawings**

21

22 Figure 1 shows the general structure of a disk centrifuge with transparent walls.

1 Figure 2 shows an end-on view of a disk centrifuge indicating the liquid meniscus and the
2 radially increasing sample band.

3 Figure 3 shows a modified disk centrifuge with one wall containing an optical berm.

4 Figure 4 shows a detector configuration based on the berm structure of Fig. 3 permitting the
5 measurement of light scattered by the sample over a range of angles.

6 Figure 5 shows a detector configuration based on the flat walled structure of Fig. 2

7 Figure 6 corresponds to an alternate form of a disk centrifuge wherein the samples are
8 contained in discrete sample cells or cuvettes.

9 Figure 7 is a schematic layout of the optical system for the Beckman Optima XL-A
10 Analytical Centrifuge.

11 Figure 8 shows a preferred embodiment of a sample cuvette incorporating a cylindrical lens
12 for use in an ultracentrifuge incorporating discrete sample cuvettes.

13 Figure 9 shows a preferred embodiment of the detection system for the Beckman Analytical
14 Centrifuge.

15 Figure 10 shows the mounting of laser and UV/visible light sources in juxtaposition to be
16 used to provide sequential illumination of the samples.

17

18 **Summary of the invention**

19 The present invention permits the sizing of particles separated by sedimentation methods,
20 such as a disk or analytical centrifuge, without requiring the use of standards for calibration.

21 Because of this capability, problems with the separation mechanisms themselves are readily
22 detected. Most centrifugation separation and subsequent sizing methods are based upon

23 measuring the intensity of a highly collimated beam of light that has passed through a sample

1 undergoing sedimentation separation. The transmitted light beam provides a measure of the
2 sample absorption as well as forward scattering. By correcting for the contributions of
3 forward scattering, assuming that the particles are homogeneous spheres and using Lorenz-
4 Mie scattering theory, and applying a size/time relation such as shown in Eq. (1), the
5 particles' effective size is derived. Most devices that use such beam geometries, such as
6 Koehler, *et al.* in their U. S. patent number 4,311,039, need to collimate the light source and
7 detector so that the detected light corresponds to that interacting with the volume containing
8 the small fraction of particles being illuminated.

9
10 The present invention modifies the detection of light passing through the sample by adding
11 optical elements, masks, and an array of detectors thus permitting measurement of the light
12 scattered by the sample over a range of angles. This multiangle light scattering detection
13 device permits the determination of the scattering particles' mean square radius that, for a
14 wide range of particle shapes, may be expressed as an effective particle size. Multiangle light
15 scattering is often referred to by the acronym MALS. From such measurement of each
16 fraction passing through the incident light beam, a size distribution may be derived that is
17 absolute and independent of the time of arrival of the sample at the detector. For many types
18 of particles in the submicrometer size range, these measurements are virtually independent of
19 both particle density and refractive index.

20
21 The inventive concepts disclosed further have immediate application to other devices
22 utilizing centrifugal forces for separation. Whereas many practitioners of such separation
23 processes have been reliant upon the use of calibration standards as well as having to make

1 the often overwhelmingly precise measurements of the physical parameters of the samples
2 and fluids involved, with the present invention the entire measurement process and
3 subsequent interpretation will be simplified significantly.

4 5 **Detailed description of the invention**

6 The typical rotor and sample containing elements of a disk centrifuge are shown in Fig. 1.
7 These include the transparent side plates **1** and **2** forming the sides of a fluid-containing
8 sample chamber therebetween and a central rotor hub **3** attached to one of the plates. A fine
9 collimated light source is shown at **4** and detector cell at **5**. Samples are introduced generally
10 through the center opening **6** of **1** via channel **7**. Such and similar structures have been
11 disclosed, for example, in the U. S. Patents 4,311,039 by Koehler *et al.* and 5,786,898 by
12 Fitzpatrick. Figure 2 shows an end-on view of the sample chamber during operation. The
13 particle sample is inserted through the opening **6** concentric with the axis of rotation **8**
14 through the rotor **3** so that it begins its separation once in contact with the fluid meniscus **9**.
15 Because of centrifugal forces, the sample migrates outwardly and eventually reaches the
16 chamber's outermost boundary **10** where it remains until the chamber is cleaned. As each
17 fraction of the sample reaches the detection region **11**, it passes through the incident light
18 beam **12** from source **4** that is monitored at detector **5**. The light beam is generally well
19 collimated and often monochromatic, for example, when the source a laser. On the other
20 hand, it may be generated by a monochromator providing a beam of controlled variable
21 wavelength. Such monochrometers are often provided as part of the apparatus of analytical
22 centrifuges. For certain classes of particles, a well-collimated light beam from a UV source is
23 preferable.

1

2 The use of such centrifuges for the determination of particle size and particle size
 3 distributions based on the type of attenuated transmitted light measurements described above,
 4 is generally referred to as the “photosedimentation method”. Because the associated
 5 separation theory refers specifically to particles of spherical shape, the versatility of the
 6 method becomes extremely limited and eventually requires for most measurements the
 7 introduction of “calibrated” standards. The departures from both theory and interpretation of
 8 arbitrarily shaped particles are rarely discussed in the literature or the patents based upon
 9 such measurements.

10

11 Although Eq. (1) is the form generally presented to show the relation between particle
 12 diameter D and arrival time t , it is instructive to examine its basis. As the chamber rotates at
 13 an angular velocity ω , a particle of mass m is forced outwardly by the centrifugal force
 14 $mR\omega^2$, where R is the distance from the axis of rotation ³. However, if the particle density is
 15 ρ_p , the fluid density is ρ_f , and the particle occupies a volume V , then the centrifugal force
 16 becomes $(\rho_p - \rho_f)VR\omega^2$. For the case of a spherical particle of radius a , the centrifugal force
 17 is simply $4\pi a^3(\rho_p - \rho_f)R\omega^2/3$. Opposing the radial motion is the so-called Stokes’ force
 18 which, for a sphere, is just $6\pi\eta a \, dR/dt$ where a is the radius of the sphere and η the viscosity
 19 of the fluid. Note that this latter formula applies only to a sphere and, therefore, the only
 20 result derivable in closed form. The net radial force on a spherical particle, therefore, is just
 21 the difference of the two forces, *i. e.*

22

$$23 \quad m\ddot{R} = 4\pi(\rho_p - \rho_f)R\omega^2 a^3 / 3 - 6\pi\eta a \dot{R} \quad (2)$$

1 or, since $m = 4\pi a^3(\rho_p - \rho_f)/3$,

2
$$\ddot{R} = R\omega^2 - 9\eta\dot{R}/[2a^2(\rho_p - \rho_f)]. \quad (3)$$

3 Thus

4
$$\ddot{R} + b\dot{R} - \omega^2 R = 0. \quad (4)$$

5 Equation (4) is readily solved in the general form

6
$$R = C_1 \exp(\alpha_1 t) + C_2 \exp(\alpha_2 t), \quad (5)$$

7 where
$$\alpha_{1,2} = \frac{-b \pm \sqrt{b^2 + 4\omega^2}}{2} = -b/2 \pm \sqrt{(b/2)^2 + \omega^2},$$

8 and $b = 9\eta/[2a^2(\rho_p - \rho_f)]$. At $t = 0$, $R = R_0$, the radius at which the sample is injected. Thus

9 $C_1 + C_2 = R_0$. Also $\dot{R} = 0$ at $t = 0$, so $C_1 \alpha_1 + C_2 \alpha_2 = 0$. Combining these initial conditions to

10 determine the coefficients C_1 and C_2 results in the final expression for the position, $R(t)$, of

11 the spherical particle as a function of time:

12
$$R(t) = R_0 \exp(-bt/2) \left[\cosh \frac{b}{2} \sqrt{1 + (2\omega/b)^2} t + \sinh \frac{b}{2} \sqrt{1 + (2\omega/b)^2} t \right]. \quad (6)$$

13

14 For typical separations for which ω is of the order of $2\pi 10^4$, where the fluid is water, the

15 sphere is of diameter 100nm, and the density difference between the particle and fluid is of

16 the order of 5×10^{-2} , the quantity $2\omega/b \ll 1$. Equation (6) then becomes simply

17
$$R = R_0 \exp(\omega^2 t / b). \quad (7)$$

18 Solving Eq. (7) for D yields Eq.(1) immediately. Note that all of these results apply to

19 spheres only and are affected considerably by even slight differences between the actual

20 particle and fluid densities and those measured. For the case of inhomogeneous particles,

1 even of spherically symmetric shape, the average particle densities well may vary with
2 particle size making the interpretation of Eq. (1) even more uncertain.

3

4 The general chamber structure of the disk centrifuge per, for example, the device previously
5 referenced by Koehler, *et al.* will result in a cylinder of fluid confined between two plates.

6 Particles confined in such rotating fluids will be subject to both centrifugal and Coriolis
7 forces. The Coriolis force, of magnitude $2\omega\dot{R}$ causes a motion in the direction of rotation.
8 Since the ratio of the Coriolis force to the centrifugal force, $\omega^2 R$, is $2\omega/b$, we see that it is
9 generally negligible.

10

11 Hoffman discloses an application of a disk centrifuge manufactured by Horiba, Ltd. of
12 Kyoto, Japan, in his U. S. Patent number 4,871,248. The Horiba disk centrifuge, *e. g.* their
13 CAPA 500, makes use of cuvettes mounted within the disk structure that rotates in a
14 horizontal plane. These small cuvettes restrict any motion in the direction of rotation and
15 eliminate thereby any Coriolis effects, no matter how great the angular velocity of the
16 system. Thus samples are placed into the cuvettes as uniform dispersions that separate into
17 specific populations during the spinning of the disk.

18

19 Light scattering is perhaps the best-known means for measuring the size of particles.
20 Measurement of the scattered light intensity, as a function of scattering angle can be used to
21 deduce such size for many diverse types of particles. For the case of a spherical particle,
22 measurement of such multiangle light scattering patterns may be used to derive both sphere
23 diameter and refractive index. Phillips, Wyatt and Berkman have demonstrated this, for

1 example, in their 1970 paper appearing in the Journal of Colloid and Interface Science,
2 volume 34, pages 159 to 162. The structure of particles exhibiting spherically symmetry may
3 also be deduced in some cases by measurement of their light scattering properties. Examples
4 of the application of MALS measurements to the determination of such structures may be
5 found in the following papers, for example:

6 "Cell Wall Thickness, Size Distribution, Refractive Index Ratio, and Dry Weight
7 Content of Living Bacteria (*Staphylococcus aureus*)," Nature **226**, 277 (1970).

8 "Dielectric Structure of Spores from Differential Light Scattering," *Spores V*,
9 American Society for Microbiology, (1971).

10 "Structure of Single Bacteria from Light Scattering," with D.T. Phillips, J. Theor.
11 Biol. **37**, 493 (1972).

12 "Some Chemical, Physical and Optical Properties of Fly Ash Particles," Applied
13 Optics **14**, 975 (1980).

14

15

16 For very small particles whose radii are less than about 10 nm, their size cannot be
17 determined by MALS for visible incident light. This limitation is due entirely to lack of
18 resolution at the wavelengths used for the measurements. Thus MALS cannot be used to
19 derive the size of proteins, for example, whose size is typically of the order of a few
20 nanometers. However, since various centrifuges, such as the analytical ultracentrifuge of
21 Beckman Instruments, are equipped with advanced absorption optical systems, they are
22 capable in principle of measuring the absolute concentration at any point in the sample. With
23 the MALS capability of the present invention, it is possible to derive protein molar masses

1 directly by combining the light scattering data with the concentration results. Historically, it
2 must be mentioned that measurements with the ultracentrifuge have been combined with
3 other measurements such as light scattering, quasielastic light scattering, and viscometry in
4 order to understand better the traditional ultracentrifuge results when the particles or proteins
5 of interest are not spheres.

6

7 When the refractive index of particles subject to MALS measurements is close to the
8 refractive index of the suspending fluid, there are several simplifications in the theoretical
9 interpretation of the MALS measurements that permit the determination of the so-called rms
10 radius of the scattering particles. For many simple structures such as spheres, rods, disks,
11 ellipsoids, etc., the rms radius may be related directly to more characteristic size parameters
12 such as radius or length. With some *a priori* knowledge of the particles' shape, the present
13 invention permits, for many classes of particles, the determination of their size even though
14 they are not spheres. In addition, for a reasonably wide range of refractive indices and
15 corresponding densities, the sizes of even spherical particles may be determined accurately
16 from their measurement in a centrifugal device incorporating the key features of the present
17 invention. As clearly evident from Eq. (1), slight errors in the determination of particle
18 density can result in large sizing errors using devices based on centrifugal separations. The
19 density of simple polystyrene spheres relative to water is only of the order of 5×10^{-2} and
20 obviously any errors in this value will have a major effect on the determination of the
21 corresponding particle size based on Eq. (1). The present invention eliminates this
22 dependency to a great extent.

23

1 In order to make MALS measurements from a sample undergoing centrifugal separation
2 according to the methods taught by the present invention, special optics and detector
3 capabilities must be incorporated into the centrifugal devices. Figure 3a shows a preferred
4 embodiment of an element of the invention. A transparent berm **12** has been integrated into
5 the outer transparent plate of a disk centrifuge. A cross section of the plate with the
6 integrated cylindrical berm is shown in Fig. 3b. In its preferred embodiment, the radius of
7 curvature of the berm will be centered at the illuminated sample.

8

9 Figure 4 shows a finely collimated light beam **13** from light beam source **4** passing through
10 the berm structure **12** and exiting at **14** before entering a transmitted light detector **15**. In the
11 preferred configuration of the optical berm, the beam **16** exits normally through a flattened
12 anti-reflection coated surface **14**. Also shown is a set of highly collimated detectors **17** each
13 receiving light scattered by the sample region **18** into unique angular directions **19** with
14 respect to the emerging beam **16**. Note that the light scattered from the sample region **18**
15 leaves the optical berm normal to its surface. The detectors **17** are collimated so as to accept
16 light scattered from the sample region **18** within a small solid angle. Because the separations
17 of particles generally span a small range of centrifugal radial distances **20**, it is essential that
18 the detector collimation provide a high degree of sample/solute resolution with respect to the
19 radial direction. This is achieved by collimation that provides for a highly localized
20 coincident field of view at each distinct detector. Adding optical lenses in front of the
21 detectors would further restrict the depth of field and, thereby, the contributing scattering
22 volume detected. The berm structure **12**, of course, may be eliminated completely resulting in
23 the emergence of scattered light through the flat outer plate surface as shown in Fig. 5. The

1 specific masking and detector orientation required to detect light scattered from the same
2 highly restricted scattering region would add some complexity to the apparatus. Most
3 importantly, the efficiency of collecting scattered light would be somewhat reduced and
4 internal reflections at the interfaces increased somewhat. Some additional masking of the
5 detectors might be needed also to minimize the passage of stray light into the detectors. In
6 the preferred embodiment of this invention, the light would be from a laser source and would
7 be plane polarized perpendicular to the plane containing the detectors 17 shown in both Figs.
8 4 and 5. Although such co-planar detectors are sufficient to provide the characterizing data
9 that would form the basis for subsequent analysis of the molecules/particles being measured,
10 for some classes of measurements or because of geometrical limitations, detectors outside of
11 such a preferred plane may be employed. Such detectors would be classified by both a
12 scattering angle and an azimuthal angle with respect to a defined plane.

13

14 The structure shown in Fig. 4 and Fig. 5 for the detection of scattered light from a disk
15 centrifuge sample is quite different from the conventional transmitted beam detection shown
16 in Fig. 1. Most importantly, the particles in the illuminated region 18 are detected by the light
17 they scatter rather than the more conventional absorption means achieved by monitoring the
18 transmitted beam. By integrating into the disk structure a transparent berm whose cross
19 section provides an optical surface normal to the scattered light, it becomes possible to
20 measure the scattered light intensity over a greater range of angles than would otherwise be
21 the case were the surface planar. For the berm structure, this range could be between 10
22 degrees and 80 degrees. Adding anti-reflection coating over the surfaces through which the
23 scattered light passes will enhance the transmission characteristics of the optical berm

1 structure further. For the case of a flat surface as shown in Fig. 5, this range is substantially
2 reduced. Thus while the flat surface simplifies significantly the construction of the sample
3 chamber, it results in a far greater reduction in the accessible scattering angle range. The
4 maximum scattering angle accessible with a flat surface is in the range of 40° to 45° which is
5 far less than the almost 80° accessible using the berm structure. For both flat and berm
6 surfaces, the forward transmitted beam 16 is measured at detector 15 which may serve also as
7 a light trap, preventing, thereby, the presence of stray light that might be scattered by
8 adjacent structures into the MALS detectors 17. The trap incorporated therein could consist
9 of a Rayleigh horn or even a mirror or prism structure that would remove the incident beam
10 and send it in a region where any light scattered from such deviated beam would not be
11 detectable by any of the collimated detectors 17. The trap could be comprised also of an
12 optically dense and non-reflecting medium such as anti-reflection coated black glass.
13 However, for measurements of the intensity of the transmitted beam 16, which for many of
14 the implementations of the photosedimentation method is required to calculate the associated
15 particle/molecular concentration, a combination of a beam intensity detector and a beam trap
16 will be required. Because of the significant refractions at the flat surface of scattered light
17 shown in Fig. 5, the angular position of the detectors relative to the incident light beam is
18 shown by the lines 19a. The actual scattering angles are indicated by the angles 19.
19
20 Once again, although the berm structure is the preferred embodiment of the sample chamber,
21 for very small particles/molecules whose scattering is close to isotropic, the flat surface and
22 its reduced range of accessible scattering angles could be used without too great a
23 degradation of the particle/molecule size derived therefrom.

1
2 Figure 6 shows the basic elements of the Horiba disk centrifuge discussed earlier. Two
3 cuvettes, containing the sample and reference fluids respectively, are placed at 21 and 22
4 within the horizontal disk 23. The light source and detector is similar to that used with the
5 Koehler *et al.* device discussed earlier though since the cells or cuvettes, and associated
6 sample, occupy only a small part of the circumference 24 traced out by the light source-
7 produced beam as the disk rotates. The light source 4 and/or detector 5 may be pulsed or
8 modulated so that they are turned on only during the period the sample or reference cuvettes
9 are in the beam. High resolution of a sedimenting sample requires that the light beam
10 diameter be as small as practical. For the Horiba device, the beam diameter is an order of
11 magnitude greater at 2 to 3 mm than that of currently available laser sources preferred for the
12 present invention.

13
14 The analytical ultracentrifuge, of the type sold by Beckman Instruments, Inc., represents a
15 device quite distinct from the disk centrifuges discussed earlier. Because they can achieve far
16 greater speeds, up to 60,000 revolutions per minute, they are able to separate far smaller
17 particles. Indeed, one of the main applications of such systems is for the study of proteins.
18 Such molecules are distinguished by their small size, rarely exceeding a few nanometers, and
19 their associated greater diffusion coefficients. The determination of molecular weight,
20 shapes, sizes, distributions and purity may, in principle, be derived directly from careful
21 measurements of various features of a sedimenting sample. Such measurements include the
22 need to observe and detect sharp boundary regions in the separating samples. The analytical
23 ultracentrifuge relations used to derive molar mass, for example, are quite different from Eq.

1 (1) used to derive the diameter of the separated particles. Rather than make assumptions
 2 concerning the molecule's shape and assuming that Stokes' law may describe the viscous
 3 drag force, the frictional force is assumed to be of the form $F_f = -fu = -f dR/dt = -f\dot{R}$, where
 4 f is the so-called frictional coefficient which depends on the particle's size and shape. The
 5 particle mass m is expressed in terms of its associated molar mass M by dividing by
 6 Avogadro's number N_a , *i. e.* $m = M/N_a$. Thus Eq. (2) is generalized to the form

$$7 \quad \frac{M}{N_a} \omega^2 R - \frac{M}{N_a} \bar{v} \rho_f \omega^2 R - f \dot{R} \approx 0, \quad (8)$$

8 where a steady state has been assumed to exist, *i. e.* $\ddot{R} \approx 0$. The second term in Eq. (8)
 9 corresponds to the contribution of buoyancy, where ρ_f is the density of the solvent and \bar{v} is
 10 the volume in g/mL displaced by each gram of the molecule. Combining the terms of Eq. (8)
 11 results in

$$12 \quad s = \frac{\dot{R}}{\omega^2 R}, \quad (9)$$

13 where $s = \frac{M}{N_a} [1 - \bar{v} \rho_f]$ is the sedimentation coefficient. For relatively sharp and
 14 symmetrical sedimenting boundaries, the sedimentation coefficient is obtained by integrating

15 $\frac{\dot{R}}{\omega^2 R}$ to yield

$$16 \quad \ln(R / R_m) = s \omega^2 t, \quad (10)$$

18 where R is the boundary midpoint and R_m is the meniscus position. Note the similarity of Eq.
 19 (10) and the corresponding result for the disk centrifuge of Eq. (7). A plot of $\ln(R)$ versus t
 20 yields a straight line of slope $\omega^2 s$ from which s may be calculated. Next, measurement of the

1 rate of boundary spreading can be used to calculate the diffusion coefficient D that will
 2 depend on the effective size of the diffusing molecules through the corresponding frictional
 3 coefficient f . Thus

$$4 \quad D = \frac{\Re T}{N_a f}. \quad (11)$$

5 The absolute temperature is T and \Re is the gas constant. Taking the ratio of the
 6 sedimentation to the diffusion coefficient using Eq. (9) and (11) gives the molar mass

$$7 \quad M = \frac{s^0 \Re T}{D^0 (1 - \bar{v} \rho)}. \quad (12)$$

8 The superscripts indicate that the calculated diffusion and sedimentation coefficients have
 9 been extrapolated to zero solute concentration. Each is generally calculated from
 10 measurements made using solutes at different concentrations.

11

12 The derivation of molar mass results based on Eq. (12) involves both a great amount of time
 13 and extensive calculations. The determination of the partial specific volume \bar{v} itself is
 14 generally no trivial matter. Indeed, the determination of molar mass, though absolute, is most
 15 difficult to measure by analytical ultracentrifuge. The preferred method by which molar mass
 16 is determined using the analytical ultracentrifuge is by means of the technique of
 17 sedimentation equilibrium. A small volume of an initially uniform solution is centrifuged at
 18 lower speeds than generally required to obtain the molar mass by means of the sedimentation
 19 velocity method of Eq. (12). This results in a concentration gradient building up from the
 20 bottom of the cuvette. The molecular diffusion increases with increasing concentration so
 21 that there are two counter flows at each concentration: a radial flow caused by the centrifugal
 22 force and an opposite flow due to diffusion. Eventually an equilibrium concentration

1 distribution is achieved where the concentration of the molecular species varies exponentially
2 with R^2 . For a monodisperse non-associating molecular solute, the molar mass may be
3 shown to be

$$M = \frac{2\Re T}{(1 - \bar{v}\rho)\omega^2} \frac{d(\ln c)}{d(R^2)}. \quad (13)$$

5 Thus a plot of $\ln c$ versus R^2 yields a slope directly proportional to the molar mass, M .

6 Alternatively, by fitting the data of c versus R^2 to an exponential using a least squares' fit,
7 one should be able to derive an estimate of $M(1 - \bar{v}\rho)$ directly.

8
9 Despite the great difficulties associated with finding molar masses directly using the
10 analytical ultracentrifuge, the power of the instrument to separate such small molecules while
11 at the same time being able study a wide range of other phenomena such as heterogeneity,
12 association reactions, and a variety of thermodynamic properties make the analytical
13 centrifuge a most useful analytical tool. Because the Beckman device measures concentration
14 directly, were the instrument combined with the elements of the present invention, its utility
15 would be extended significantly. Thus molar masses could be calculated directly by
16 combining concentration measurements with an absolute measurement of light scattered by
17 the samples being studied. Once molar masses were so easily obtained, more accurate values
18 of both sedimentation and diffusion constants could be derived almost effortlessly. The
19 means by which the preferred embodiment of the present invention may be applied to the
20 analytical ultracentrifuge will now be discussed.

21
22 Figure 7a presents a schematic of the optical system of the Beckman analytical
23 ultracentrifuge. The sample holding rotor **25** rotates about shaft **3** within an evacuated

1 chamber. Similar to the Horiba structure of Fig. 6, the rotor contains sample and reference
2 cuvettes. However, two pairs are included at diametrically opposite locations **26** and **27**.
3 Figure 7b shows a top view of one of these locations, for example **26**, containing sample **28**
4 and reference **29** cuvettes. By this means, two distinct samples may be processed during each
5 experiment. Note that each cuvette is constructed with a side boundary lying along a radius at
6 a slight angle to the other side that lies along the principal diameter of the rotor. This
7 structure helps reduce internal sample streaming during separation. A Xenon flash lamp
8 source **30** is shown together with a steering diffraction grating **31** and incident light monitor
9 **32** that receives a small signal proportional to the incident intensity by means of a beam
10 splitter **33**. The focused beam **34** passes sequentially through the sample and reference cells
11 when cell-containing regions **26** or **27** are in the beam. In general, the incident light source is
12 flashed so that the beam is on only during its passage through the sample or reference cells.
13 The diffraction grating permits also the selection of the wavelength of the incident beam over
14 the range of possibilities associated with the light source; in this case, a Xenon lamp.
15 Generally wavelengths in the near ultraviolet are selected as a great many studies with such
16 apparatus involve proteinaceous materials that absorb strongly in the UV. An optical imaging
17 system **35** collects light transmitted through the sample within **26** or **27** from a small radial
18 region within the sedimenting sample. The image of the mask **36** at the illuminated sample
19 defines this narrow field of view. A photodetector such as a photomultiplier tube **37** detects
20 this transmitted light, though other detection systems may be employed.
21
22 The structure comprised of the beam **34**, imaging system **35**, and photodetector **37** are
23 controlled to move in the radial direction as a unit permitting, thereby, the sample to be

1 measured at different radial distances. For the disk centrifuges, on the other hand, the
2 beam/detector pair is set at a single radial distance throughout the entire measurement.
3 Depending upon the types of particles/molecules to be separated, the radial scans are
4 repeated many times to yield a sedimentation profile as a function of time. In the preferred
5 embodiment of this invention, when elastically scattered light measurements are
6 implemented, the preferable light sources will be lasers. The optical systems can
7 accommodate both UV flashed sources as well as lasers so that both concentration and so-
8 called static light scattering measurements may be made almost simultaneously.

9
10 Unlike the disk centrifuge of Figs.1 through 5, the analytical ultracentrifuge and the cuvette
11 based disk centrifuge cannot benefit from the circular berm lens structure disclosed earlier. In
12 their preferred embodiment, the individual cuvettes *per se* must incorporate the required
13 cylindrical lens structure. They may be of the simpler flat surface construction, as well;
14 however the range of accessible scattering angles is minimized because of the surface
15 refractions. In addition as the refractions approach the critical angle, the intensity of the
16 transmitted scattered light becomes greatly attenuated. Nevertheless, for smaller
17 particles/molecules, the reduced range of scattering angles accessible with a flat exit surface
18 may be sufficient to derive their corresponding sizes. For very small particles/molecules
19 Figure8 presents a section of a cuvette that could be used in the preferred embodiment of the
20 present invention described in the parent case serial 10/202,777. Instead of having flat sides
21 through which the light beam will pass, the flat surface through which the beam exits has
22 been modified to provide a cylindrical structure 38 whose radius of curvature, in the
23 preferred embodiment, is centered at the illuminated sample. For the ultracentrifuge, the

1 beam also may be scanned radially along the length of the cuvette in the direction of
2 increasing radius 39; while for the disk centrifuge incorporating such cuvettes, the beam will
3 be restricted to a fixed position. This cylindrical surface of the preferred embodiment may
4 have a flattened apex 40 corresponding to the similar structure for the berm. The entire
5 region may be antireflection coated, as well. Once again, the standard flat cuvette may be
6 used to make measurements over a restricted range of scattering angles.
7
8 Figure 9 shows an instantaneous cross sectional view of the preferred embodiment for an
9 analytical ultracentrifuge. Also shown are the imaging system 35 and the photomultiplier
10 detector 37 of the conventional structure of Fig. 6a. In the region between this imaging
11 system 35 and the rotor housing 25 are a set of collimated scattered light detectors 41 similar
12 to the detectors 17 of Fig. 4. These detectors, as well as those shown in Fig.4, generally lie in
13 a plane and intercept scattered light from a small illuminated volume 38 within the
14 cuvette/cell 28. They move with the other elements of the imaging system and collect
15 scattered light throughout the radial scanning transverse to the plane of the figure. Thus the
16 sample particle mass and size distribution profiles throughout the scanned cuvettes may be
17 determined from the scattering measurements and recorded by the inventive system
18 described. The light beam 34 is therefore always perpendicular to the imaging system 35 and
19 the photomultiplier tube 37 to which are attached the scattered light detectors 17. Thus all
20 detection, light sources, and optical elements are on a fixed platform relative to the rotor 25.
21 The platform may move radially, permitting, thereby, the radial scanning capability of the
22 analytical ultra centrifuge. The ability to make radial scans during operation of conventional
23 disc centrifuges is a feature not seen with such devices, but is relatively easy to implement.

1 When the reference cuvette **29** arrives at the position previously occupied by the sample
2 cuvette **28**, light scattered by its solution will be collected in a similar manner.
3

4 The light source producing the beam incident upon the sample and reference cuvettes can
5 originate from a laser source or, alternatively, from a variable UV/visible light source such as
6 commonly used in the analytical ultracentrifuge. On the other hand, both variable and laser
7 sources may be activated sequentially or simultaneously. In Fig. 10 a laser source **42** used to
8 produce a light beam to interrogate the sample is shown mounted in juxtaposition to the
9 steering diffraction grating **31**. Both lie at the same radial distance, but displaced in rotation
10 angle. The UV source beam **34** from the Xenon flash lamp **30** will strike the cuvette after the
11 incident the laser beam **43** has passed through it as the cuvette rotates counterclockwise
12 through the same radial position with respect to the cuvette-contained separating sample **28**.
13 The corresponding transmitted UV/visible light beam **34** intensity, used to calculate the
14 sample absorption, and the light scattered from laser beam **43** into detectors **17**, are combined
15 to calculate size and mass. The signals UV and laser beam interactions with the sample are
16 collected sequentially at the same radial position of the sample. Note that the UV/ visible
17 light source itself has an associated set of scattered light detectors **41** to detect scattered light
18 from the sample. The UV/ visible light source may be replaced equivalently by any other
19 light source or sources and used, for example, with a photodiode array detector.
20

21 Alternatively, the laser beam may be arranged to be collinear with the UV/ visible light
22 source, or to replace it if the determination of the molar mass using concentration detection
23 means is not required. A multiwavelength laser or light source may be selected as well, with

1 various filters chosen to select the transmitted beam wavelength. Still other filters may be
2 selected and attached to the scattered light detectors to eliminate detection of specific
3 scattered wavelengths. There are many other means for providing such beams, as would be
4 obvious to those skilled in the art of optical design

5
6 The dual source geometry discussed above for the case of the analytical ultracentrifuge,
7 whereby the sample is illuminated sequentially by the two different light sources as the
8 sample containing region rotates past them, may be applied as well to the disk centrifuge
9 geometries discussed earlier. This dual sequential illumination may be implemented with the
10 structures of Figs. 2, 4, or 6. Indeed, multiple illumination sources may always be used for
11 any of the centrifugal separation devices. Such multiple sources are not restricted to two.

12
13 If the laser and UV/ visible light sources were oriented so that they formed a single incident
14 beam, then the scattered light detectors 17 would be fitted preferably with filters to remove
15 UV/ visible light scattered by the solutions. Such filters would be preferably interference
16 filters permitting only elastically scattered light at the laser wavelength to be detected. If
17 inelastically scattered laser light is to be detected, such as caused by fluorescence, the
18 corresponding detector filters would be selected accordingly. If any of the light sources
19 employed is polarized, polarization sensitive analyzers may be attached to selected scattered
20 light detectors to permit quantitative measurement of depolarization scattering effects.

21
22 It is clear from the discussions above that the key elements of this invention apply equally to
23 various types of instrumentation using photometric means to monitor sedimentation

1 phenomena induced by an applied centrifugal force. The purpose of the invention is to permit
2 the measurement of scattered light from regions of the sample being separated by such
3 means. From such measurements made over a range of scattering angles, it becomes possible
4 to derive particle size directly, irrespective of diffusion phenomena. For the case of sub-
5 micrometer particles, means by which such scattered light measurements may be used to
6 measure particle size and size distributions has been explained in such papers as:
7 “Absolute Measurement of Diameter Distributions of Particles Using a Multiangle Light
8 Scattering Photometer Coupled With Flow Field-Flow Fractionation,” D.W. Shortt and D.
9 Roessner, and P. J. Wyatt, *Am. Lab.* **28** 17, 21 (1996); and
10 “Submicrometer particle sizing by multiangle light scattering following fractionation,” P. J.
11 Wyatt, *J. Colloid and Interface Science* **1979**, 9-20 (1998).

12
13 For the case of solvated molecules undergoing separation by centrifugal means, the weight
14 average molar mass may be derived directly if the concentration of the molecules be known
15 in addition to the differential refractive index increment, dn/dc . Details of such quantities
16 may be found in the co-pending application by Wyatt and Trainoff referenced at the
17 beginning of this specification as well as the reference by Wyatt discussed in the next
18 paragraph. Most centrifugal separation devices, and certainly the analytical ultracentrifuge,
19 use a light beam whose absorption by the solution may be used directly to calculate the
20 concentration of the molecules present. Thus for the case of solvated molecules, a UV/
21 visible light source is often sufficient as it exists to produce absorption data sufficient to
22 determine, from the sample’s extinction coefficient, the molecular concentration. For larger
23 particles, on the other hand, such absorption techniques rarely may be used to calculate the

1 particle concentration because of the role played by the particle scattering. In addition, the
2 angular variation of such particle scattering is generally sufficient to calculate the effective
3 particle size. Since the forward transmitted beam that passes directly through the sample is
4 useful to determine the molecular concentration and, perhaps for some particles, the beam
5 transmittance, the preferred embodiment of the invention would continue the use of such
6 measurements.

7
8 The ability to measure molecular mass and size directly for samples undergoing centrifugal
9 separation, and especially for proteins, is a particularly significant application of this
10 invention as it potentially eliminates those elements most difficult to measure from
11 conventional analytical ultracentrifugal analysis. Most important among such elements is the
12 determination of the volume of solvent displaced by the molecule whose mass and size is to
13 be determined. Once the concentration and light scattering response as a function of
14 scattering angle of a particular molecular species are known, the molecular mass may be
15 determined immediately following the methods described in detail by Wyatt in his 1993
16 *Analytica Chimica Acta* paper in volume 271, pages 1 *et seq.*, entitled "Light Scattering and
17 the Absolute Characterization of Macromolecules." Once the mass of a separating species
18 has been so-determined, the molecules' volume, for example, may be calculated explicitly
19 from the sedimentation coefficient derived via Eq. (10). Such determinations have never been
20 made directly in this manner. The implications of these direct determinations for the protein
21 chemistry and related fields are of great importance.

22

1 Another key element of the invention relates to the modifications required of the exit surfaces
2 of the sample-containing regions. As the beam leaves the sample, it should exit preferably
3 normal to the transparent region in which the sample is restricted. Thus the normal surface
4 through which the undeviated incident beam passes remains the same as that currently
5 employed in such centrifugal separation devices. A major modification of the surface region
6 is required so that the light scattered from the sample can be measured also over a reasonable
7 range of angles. As has been mentioned previously, the invention is intended to permit
8 measurement of light scattered over a range of scattering angles from a small volume within
9 the illuminated sample. Thus scattered light leaves the sample-containing surface normal
10 thereto in its passage to a set of detectors placed preferably at equidistant radial positions
11 from the small scattering volume. The collimation of these detectors defines the field of
12 view, *i. e.* the transverse dimension of the scattering volume. The optical surface itself,
13 generally forming a cylindrical lens whose apex has been flattened as described previously,
14 would have its radius of curvature centered at the illuminated sample volume. A flat surface
15 may be employed if the range of accessible scattering angles may be restricted.

16
17 As will be evident to those skilled in the arts of light scattering, there are many obvious
18 variations of the modified centrifugal separators I have invented and described that do not
19 depart from the fundamental elements that I have listed for their practice; all such variations
20 are but obvious implementations of my invention described hereinbefore and are included by
21 reference to my claims, which follow.

22

23